FREE FAECAL LIQUID IN HORSES – CHEMICAL COMPOSITION OF FAECES IN CASES AND CONTROLS

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Tiivistelmä — Referat — Abstract

Some horses experience a problem called free faecal liquid (FFL). The condition is not characterized by classical diarrhoea but by free liquid that is voided separately from the solid part of the faeces. Problems with FFL include irritation and abrasive lesions in the skin in the rear end. No health issues of more severe character are usually present in these horses, apart from preliminary findings indicating a higher risk of colic when compared to horses without FFL. The reason for why horses develop FFL, and its effects on the horse, are unknown as the subject has not been studied much. It has been suggested that one reason could be in how the horses are fed. It has been speculated that haylage could have an impact on the development of FFL.

A study was done with the aim to collect information about feeding and management of horses affected with FFL in Sweden and Norway (n = 100), and to compare faecal composition in horses with and without FFL. This particular part of the study focused on the faecal properties. The objective was to compare chemical composition of the faeces of affected and unaffected horses, in order to detect possible reasons for FFL, which could be studied further. The study was performed with three repeated samplings on all horses. Case and control horses were paired and were housed at the same farm and were fed the same forage. The horse owners were collecting faecal samples using a standardized protocol. The variables examined in faecal samples were dry matter (DM), pH, volume of free liquid after centrifugation, sand, osmolality, acetate, propionate, isobutyrate, n-butyrate and total volatile fatty acid (VFA) concentration of the samples. In samples from the first sampling occasion, differences ($P < 0.05$) between cases and controls were found in concentration of acetate, isobutyrate and total amount of VFA. Tendencies toward differences ($0.10 < P > 0.05$) were found in the volume of liquid and sand as well as in n-butyrate concentration. At the second sampling tendencies toward differences were found in the amount of sand and in n-butyrate concentration. In samples from the third sampling a tendency toward difference was present for pH value. All values were or tended to be higher for horses with FFL. Also, clear correlations were found between nearly all the variables. The pH value was found to correlate negatively with osmolality and all the VFAs except for isobutyrate where no correlation was found in any of the sampling occasions. Osmolality was also found to correlate positively with all the VFAs. VFAs correlated positively with one another.

The results showed a tendency toward difference in n-butyrate concentration and presence of sand in faecal samples when case and control horses were compared, although not with consistency as samples form the third sampling occasion did not follow results from the first and second samplings. As the results were not constant throughout the study, these two variables may not be reliable when trying to find possible reasons for FFL. Almost all the variables were found to correlate with one another, exception being isobutyrate with pH. As butyrate is a major source of energy for horses and important for the health of the equine intestine, this information could be useful in further studies concerning the possible reasons for FFL.

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Equine, FFL, haylage, faecal composition

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Tiivistelmä — Referat — Abstract

Joillain hevosilla on havaittu vapaata nestettä ulosteen mukana (free faecal liquid, FFL). Klassinen ripuli ei yleensä ole tunnusomainen merkki tälle tilalle, vaan uloste on kaksivaiheista; neste on erillään lannan kiinteästä osasta. FFL voi aiheuttaa ongelmia kuten ärsytystä ja haavaumia hevosen takapäässä. Vakavampia terveysongelmia ei yleensä tavata FFL yhteydessä, poissulkien alustavat havainnot FFL-hevosten mahdollisesti kohonneesta ähkyriskistä oireettomiin hevosiin verrattuna. Syyt FFL kehittymiseen ja sen vaikutukset hevoseen ovat toistaiseksi tuntemattomia, sillä ongelmaa ei ole tutkittu paljon. On ehdotettu, että hevosen ruokinta voisi olla yksi vaikuttava tekijä. On myös mietitty, että säilöheinällä voisi olla vaikutusta FFL kehittymiseen.

Tutkimus tehtiin tavoitteenaan selvittää, millaista FFL-hevosten ruokinta ja ylläpito on Ruotsissa ja Norjassa (n = 100). Myös lannan koostumusta vertailtiin FFL-hevosten ja oireettomien hevosten välillä. Tämä kyseinen tutkimuksen osa keskittyi lannan koostumuksen tutkimiseen. Tavoitteena oli verrata ulosteen kemiallista koostumusta FFL-hevosten ja oireettomien hevosten välillä, jotta mahdollisia syitä tälle terveysongelmalle voitaisiin määrittää ja tutkia jatkossa. Näytteenottotilanteita oli kolme. Tapaus- ja verrokkihevoset olivat pareissa ja yksittäiset parit elivät samassa tallissa samalla ylläpidolla, syöden myös samaa rehua. Hevosten omistajat keräsivät näytteet standardoitua protokollaa käyttäen. Muuttujat, joita tarkasteltiin, olivat kuiva-aine (KA), pH, vapaan nesteen määrä sentrifugoinnin jälkeen, hiekka, osmolaliteetti, asetaatti, isobutyraatti, n-butyraatti ja haihtuvien rasvahappojen (VFA) kokonaismäärä näytteissä. Ensimmäisen näytteenottokerran näytteissä eroavaisuuksia ($P < 0.05$) tapaus- ja verrokkihevosten välillä havaittiin asetaatin, isobutyraatin ja haihtuvien rasvahappojen kokonaispitoisuudessa. Suuntaa antavia eroavaisuuksia ($0.10 < P > 0.05$) havaittiin vapaan nesteen määrässä, hiekassa sekä n-butyraatin pitoisuudessa. Toisessa näytteenottokierroksessa huomattiin suuntaa antavia eroavaisuuksia hiekan määrässä ja n-butyraatin pitoisuudessa. Kolmannella näytteenottokerralla havaittiin suuntaa antava eroavaisuus pH-arvossa. Kaikki arvot, joissa huomattiin eroavaisuus tai suuntaa-antavuus, olivat korkeampia FFL-hevosilla. Selviä korrelaatioita havaittiin lähes kaikkien muuttujien välillä. Osmolaliteetin ja kaikkien haihtuvien rasvahappojen lukuunottamatta isobutyraattia havaittiin korreloivan negatiivisesti pH-arvon kanssa kaikilla näytteenottokerroilla. Osmolaliteetin havaittiin myös korreloivan positiivisesti kaikkian haihtuvien rasvahappojen kanssa. Haihtuvat rasvahapot korreloivat positiivisesti toistensa kanssa.

Tulokset viittaavat siihen, että n-butyraatin pitoisuudessa ja hiekan määrässä oli eroavaisuuksia tapaus- ja verrokkihevosten välillä, vaikkei tulokset olleetkaan yhtenäisiä; kolmannen näytteenottokerran tulokset erosivat tässä kahdesta ensimmäisestä. Koska tulokset eivät olleet yhtenäisiä läpi tutkimuksen, eivät nämä kaksi muuttujaa välttämättä ole luotettavia, kun syitä FFL:n taustalla yritetään selvittää. Lähes kaikki muuttujat korreloivat keskenään, poikkeuksena isobutyraatti pH-arvon kanssa. Koska butyraatti on tärkeä hevosen ruoansulatuskanavalle, tätä tietoa voitaisiin hyödyntää tulevissa FFL-tutkimuksissa.

Avainsanat — Nyckelord — Keywords
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ABBREVIATIONS AND SYMBOLS

FFL	free faecal liquid
VFA	volatile fatty acid
DM	dry matter
Rpm	rounds per minute
HPLC	high performance liquid chromatography

1 INTRODUCTION

Silages are forage materials that then have undergone anaerobic lactic acid bacterial fermentation (Dryden 2008). Haylage is a kind of silage but with a lesser water content (Harris *et al*. 2017). Traditionally horses in Sweden have been fed with hay as their roughage, but nowadays silage and haylage are fed to horses in an increasing amount and approximately 50 % of all horses have silage or haylage in their diets (Sundberg *et al*. 2008; Enhäll *et al*. 2012). Wrapped forages have partially or totally replaced hay in many countries, including Finland and Sweden (Uotila *et al*. 2012; Müller 2018). The proportion of haylage of the total roughage is often higher for hard training gallop and harness racing horses as well as horses in riding schools and for horses in stables providing riding tours than for other horses in Sweden (Enhäll *et al*. 2012). A study was done in Finland to see how commonly forage feed analysis are used and how important they are considered to be (Uotila *et al*. 2012). In this study, stable owners got also to answer the question of which forage was given to the horses in the stable. According to this study, one fourth of the stables used hay, another fourth haylage and the rest used both forages in combination. Considering the results, haylage is commonly used also in Finland.

Roughage is the basis of a horse's diet. People feeding their horses silage and haylage instead of or in addition to hay can have a connection to the fact that hay can be dusty and mouldy at times, which can cause health issues in horses. There could for instance be mould growth in the hay because of humidity. Another reason for problems, especially airway problems in horses, is the fact that hay is dry and oftentimes dusty, spreading mould spores (Sundberg *et al*. 2008). It is thought that horses may be more sensitive towards the negative effects of mycotoxins than for example bovines, being monogastric and non-ruminant. Only little is known, however, except for the fact that horses are especially sensitive towards fumonisins. Other mycotoxins have been studied less in horses and their effects are therefore less known (Liesener *et al*. 2010). It has been suggested that factors related to feed, such as mycotoxins, may play a part in developing

FFL as they can affect the microbial population of the intestine and may cause a low-grade inflammation of the intestinal mucosa (Ertelt and Gehlen 2015).

Free faecal liquid (FFL) has been noticed of some equine individuals and it is thought that haylage could be a factor in this problem. As the name of the problem suggest, the symptom is that the faeces are divided in two separate parts: there is a solid and a liquid part of the faeces. This kind of faeces makes the rear end of the horse unclean and can in severe cases even cause skin lesions around the anus and on the hindlegs (Kienzle *et al*. 2016). It should however be noticed that haylage isn't the only suggested explanation for horses to develop FFL. Factors such as eating too much alfalfa hay, drinking really cold water, parasites and dental problems have also been suggested to play a part in developing the problem. While the exact reason for developing FFL is not known, it is currently being studied. Oftentimes it is only the digestive system that shows noticeable symptoms, in other words FFL (Kienzle *et al*. 2016). Therefore, it could be thought that the problem lies in the gastrointestinal system and thus could be related to feeding. For example, studying the contents of faeces could give clues about reasons behind FFL.

2 FREE FAECAL LIQUID IN HORSES

2.1 The equine gastrointestinal system

Horse's digestion begins with mastication which can be divided into three phases: opening phase, closing phase and power stroke. Breaking the fibre down into smaller particles is important for later digestion but also for the releasing soluble nutrient components that can be digested pre-cecally. Unlike many other species, horses' saliva mainly acts as a lubricant for the consumed feed because of the low amylase activity. The amount of secreted saliva is large. Equine saliva is mainly composed of water and has a rather high content of calcium and chlorine (Coenen *et al*. 2013).

From mouth the feed mass enters the oesophagus. The oesophagus of an average adult horse of 500 kg is about 1,2-1,5 meters long. It has a sphincter in both ends and can be

divided into cervical, thoracic and abdominal parts. The movement of digesta through the oesophagus occurs via muscle contractions. About two thirds of the equine oesophagus is striated. In this part the contractions are faster but shorter in duration. The distal third of the oesophagus consists of smooth muscle. The mucosa lining the oesophagus has no considerable secretory activity (Coenen *et al*. 2013).

Equine stomach consists of an oesophageal part, cardia, fundus and corpus as well as pylorus (Sjaastad *et al*. 2010; Coenen *et al*. 2013). The equine stomach is small in size, only about 8 % of the total digestive system. This means that in an average full-grown horse of 500 kg the size of the stomach is approximately 8-15 litres. The gastric bacteria population is varied and active. It turns most of the non-structural carbohydrate-based nutrients into lactic acid and a smaller part of it to volatile fatty acids (VFA). Composition and amount of feed affects the rate of emptying, liquids leaving the stomach faster than solids. Passage rate of concentrates and forages varies (Coenen *et al*. 2013).

The equine small intestine has three parts as does the small intestine of other mammals: duodenum, jejunum and ileum. Small intestine of the horse is quite short relative to the animal's body size, the longest part being the jejunum. The small intestine is lined with mucosa composed of villi which are each surrounded by crypts. Secretions aiding the digestion that are released into the small intestine origin mainly from liver and pancreas. Also, a number of anaerobic and facultative aerobic bacteria is present. There is not, however, data on their involvement in carbohydrate, fat or protein digestion. The motility of the equine small intestine is somehow similar to other species', it having three phases. Peristaltic and segmentation movements of phase II control transit of contents. All of this is regulated by the enteric nervous system (ENS) (Coenen *et al*. 2013).

Both cecum and colon are involved in the fermentation process in the equine intestine, making it a hindgut fermenter. Anaerobic environment, limited changes of the pH value and fast absorption of the fermentation products are important for the fermentative process (Coenen *et al*. 2013). This applies also to steady delivery of contents into and through the system (Fraser 2010; Coenen *et al*. 2013). There are several different types

of fungi, bacteria, archaea and protozoa participating in the fermentation process. Together these microbes turn the carbohydrate-based contents into volatile fatty acids (VFA). Sometimes also lactate is produced. There is limited data on the motility of the equine cecum and colon, but it seems to be complex and it is critical for good digestion and absorption of water in this part of the gastrointestinal system (Coenen *et al*. 2013). It is known that the horse's hindgut microflora is extremely reactive, and abrupt changes in horses' diet have been seen to cause rapid microbial changes in the cecum and colon (de Fombelle *et al.* 2001). No short-term effects were detected in the colon ecosystem when grass hay was changed to grass haylage or grass silage with an introduction period of three weeks in a study by Muhonen *et al*. (2009). Though it has to be noted that it was not a question of change in feed as it came from the same harvest, but it was a change of conservation method.

Not only are the microbial populations of the equine gastrointestinal track different when compared to each other; horses are also known to have great individual variation when it comes to the microbiota of their gastrointestinal system (Schoster *et al.* 2013). This understanding of the unique function of each horse's digestion is crucial when planning an individual horse's feeding.

2.2 Volatile fatty acids

VFAs are produced in the horse's gastrointestinal tract by bacterial fermentation. The primary fatty acids produced are acetate, butyrate and propionate. These three make up approximately 97 % of all the VFAs produced. The rest is other VFAs; valerate and isovalerate. By altering the horse's diet, for example by changing the grain/concentrate rations, the VFA composition can be affected (Coenen *et al*. 2013). For example, in one study plasma acetate concentration has been seen to increase in lactating mares when fed with 95 % hay and 5 % concentrate diet when compared to a diet with 50 % hay and 50 % concentrate (Doreau *et al.* 1992).

A big portion of the butyrate is utilized by enterocytes. In the liver this VFA is converted into β-hydroxybutyrate. Propionate on the other hand is used for gluconeogenesis in the liver and is responsible for much of the horse's endogenous glucose production. These both are also vital providers of oxidizable carbon for the peripheral tissues. Acetate is likely also important for the peripheral tissues as it could be a major fuel substrate for these tissues (Coenen *et al*. 2013). In one *ex vivo* study it was seen that acetate was the most important source of carbon for fatty acid synthesis in horses rather than glucose (Suagee *et al.* 2010).

2.3 Free faecal liquid

Free faecal liquid (FFL) in horses has not been studied much so far. The condition is not characterized by classical diarrhoea but rather – as the name suggests – by faecal liquid that comes out separated from the solid part of the faeces (Kienzle *et al*. 2016). This could happen before, after or during defecation (Valle *et al*. 2013). Classical diarrhoea on the other hand can be described as frequent loose or liquid bowel movements (Alexander 1978; Kienzle *et al*. 2016). The condition can be life-threatening (Kienzle *et al*. 2016). Problems caused by FFL include unclean rear end which is not only a cosmetic downside but can also lead to dermatitis and even skin lesions around the anus and on the hindlegs (Valle *et al*. 2013; Kienzle *et al*. 2016; Gerstner and Liesegang 2018).

Some risk factors possibly contributing to the process of the horse developing FFL were charted out in a study by Kienzle *et al*. (2016). This study found no relevant issues in management of the horses that could be causing the problem, though the researchers still stuck with their hypothesis of certain roughages affecting the development of free faecal liquid when paired with a insufficiently functioning digestive tract.

In the Kienzle *et al.* (2016) study it could be seen that factors such as low rank in the social hierarchy, being a paint and being a gelding could be affecting the development of FFL. According to the researchers' speculations these three could be bundled together in the sense that both geldings and paints are usually dominated by adult mares in a group

of horses and are therefore lower in the hierarchy. According to Kienzle *et al*. (2016) this status in the group could cause stress in some individuals because of threats from the dominant animals and because for example space might be limited for the horses of a lower rank when avoiding the more dominant animals. It is also important to note that factors such as stress and nervousness, which could be caused by a low rank in the social hierarchy, often affect the horse's eating behaviour as it gets to eat last and possibly less and could that way affect the horse's gastrointestinal system (Zeyner *et al*. 2004; Kienzle *et al*. 2016).

Kienzle *et al*. (2016) also created two major questions concerning FFL: to why diarrhoea is not present, but the faeces come in two phases and why the health status of horses suffering from FFL is not severely lowered. It is discussed that the particle network of the faeces is changed permanently because of too much mechanical pressure in the intestine while in horses unaffected by FFL the liquid in the faeces will go back to gel form when the pressure in the intestine is released (Lentle and Janssen 2010; Kienzle *et al*. 2016).

As the reason behind FFL is unknown, there are not any standardized recommendations regarding treatment and management of horses showing FFL (Gerstner and Liesegang 2018). One case of a horse suffering from both chronic diarrhoea and FFL was shown to benefit from adjusting the feeding management and by treating the horse with sulfasalazine. These both seemed to improve the faecal quality. The nutritional changes in this particular case included feeding the horse with high-quality first-cut meadow hay as well as ground and pelleted meadow hay and restricting the amount of starch in both forage and concentrate as high-starch diet has been shown to be a risk factors when it comes to certain problems in the equine digestive system, such as lesions on the mucosal surface in the stomach and large intestine (Valle *et al*. 2013). It should be noted that only one horse was examined in this study and therefore it cannot be assumed that this treatment would work in other cases of FFL.

2.4 Haylage

Haylage is defined as a low-moisture grass silage that has a DM content of typically 50-70 % after being wilted or dried in the field but the moisture content has been noted to be as high as 85 % (Finner 1966; Harris *et al*. 2017). Haylage is conserved by air-tight storage rather than fermentation, which makes the anaerobic seal crucial for the conserving process (Kuoppala *et al*. 2012). The fermentation of haylage is therefore restricted and it is due to the high dry matter content when compared to silage. Haylage has also been noted to have higher values of pH as well as lower concentrations of organic acids than silage (Müller 2005). The anaerobic seal is important throughout the whole storage period to assure the hygienic quality of the product as well as to avoid the heating of the feed (Kuoppala *et al*. 2012). Thus, the primary preservation method of haylage is by a combination of airtight storage and drying, not by ensiling (Müller 2018).

Horses are grass eaters and hindgut fermenters which makes roughage the most important part in their diet (Coenen *et al*. 2013). Traditionally horses in Scandinavia are fed hay but sometimes problems with mouldy or dusty hay are experienced (Sundberg *et al*. 2008). Problems could be caused by moisture content that is too high. This could occur for example because of humidity or changes in temperature. Especially during winter this can be an issue. Both animals and humans could suffer from health issues when in contact with mould contaminated hay. This and the fact that haylage is less dusty than hay are the reasons why sometimes an alternative with a higher moisture content than hay is preferred. As long as haylage is properly wrapped in plastic, it has a smaller risk for mould growth than hay. When compared with silage, the risk can be higher (Seguin *et al*. 2010; Sundberg *et al*. 2008; Müller *et al*. 2011). Some horses may also prefer a feed with a higher moisture content. In a study by Müller and Udén (2007) it was noted that horses prefer haylage over hay, which suggest that haylage can be a more palatable choice of feed. Though it was also noted during the study that most of the test horses preferred silage over haylage, which also further supports this theory. Müller and Udén (2007) speculated that this could be because more moist feed resembles grass more than dry hay does. Though the moisture content of the forage has an impact on the feed quality, it is

suggested that it is not the most important part when it comes to affecting the horse's microbial and chemical composition in the colon and faeces. (Müller *et al*. 2008).

The harvest time of all forages affect the nutrient content of the forage. It has been seen in studies that late harvested haylage contains less nutrients and has a lower dry matter digestibility than early harvested haylage, that in turn has been suggested to have a higher fermentability (Brøkner *et al*. 2010; Müller 2012). It is therefore implied that haylage from a late harvest could be a suitable feed for horses on maintenance or for horses on light to moderate exercise, while haylage from an early harvest had a higher digestibility as well as higher content of VFAs in the faeces (Müller 2012). Haylage from an early harvest has also been noted to have a lower amount of lignin and fibre than haylage from a later harvest. Late harvest date may also result in longer eating time. This is partly due to the longer eating time per kg dry matter but also the fact that the amount of feed must be increased to cover the energy and nutrient needs of the horse while these levels are lower in late harvest haylage (Müller 2011).

The time spent eating per day is important as food anticipation, with other words the stress caused by waiting for the feed, may cause stress and stereotypic behaviour in horses. This applies not only to time spent eating but also number of feeding times per day. It might even be that fewer feeding times per day cause less stress because feed anticipation is experienced fewer times a day. On the other hand, *ad libitum* feeding result in no feed anticipation at all (McGreevy and Nicol 1998; Bachmann *et al*. 2003).

3 OBJECTIVE

This study is a part of a larger study examining the presence of free faecal liquid in horses in Sweden and Norway, and if any specific feeding and management routines contain any risk factors for FFL. Haylage feeding is in focus in this study as it has been suggested to be a possible reason in horses developing FFL. This sub-study focuses on comparing the composition of faeces from horses with and without FFL, and includes biochemical

composition. The aim of the study was to compare faecal composition between case and control horses, in order to map if differences are present. If so, differences may provide clues to causes of FFL.

The hypothesis is that there are differences between the control group and case group in the microbial and biochemical composition of the faeces.

4 MATERIALS AND METHODS

4.1 Study participants and samples

For the study, 300 faecal samples were collected in Sweden and Norway. All together 100 horses participated in the study, and these were sampled thrice. Of the horses 60 were from Sweden and 40 from Norway. Of the horses 49 were mares and 51 were geldings. No stallions were included in the study. The gender of the horses is presented by country in Figure 1. The ages of the participating horses varied between two and 26 years and the age statistics are presented in Table 1.

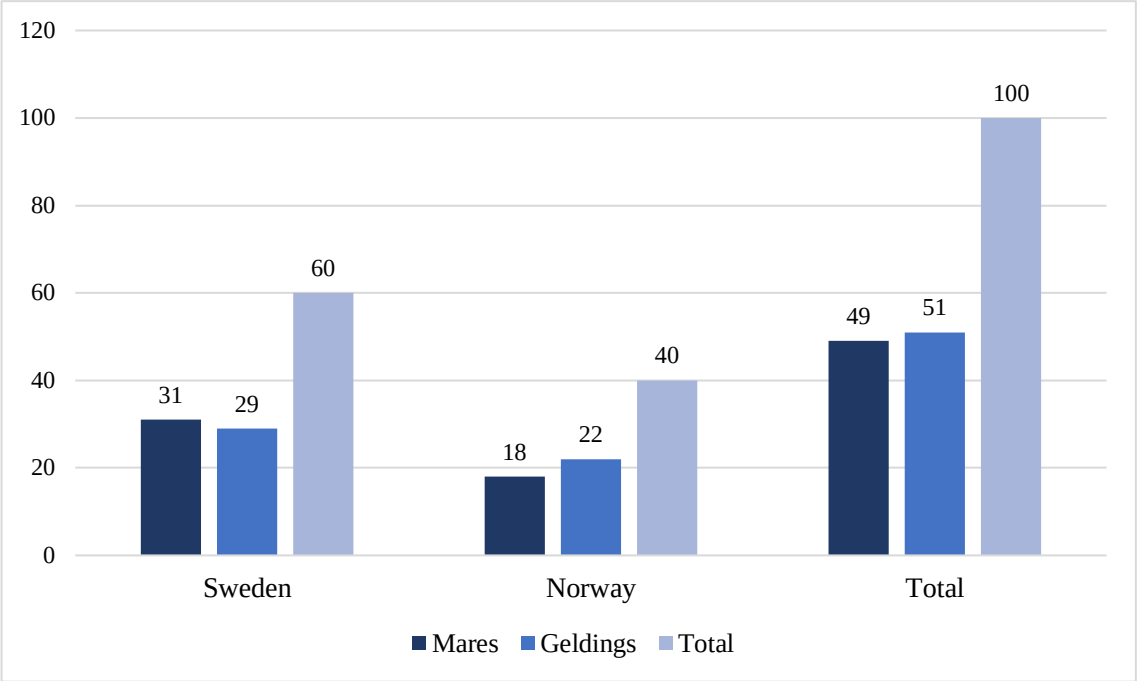

Figure 1. Gender of the participating horses by country and total number of horses in the study.

Table 1. Age statistics of the participating horses

	Sweden	**Norway**	**Total**
		age in years	
Mean	11,9	10,6	11,4
Median	11	10	10
Min	2	2	2
Max	26	21	26

In the study there was a total of 27 different horse breeds and there was not an equal amount of horses of every breed. There was also variation in the breeds between the two countries. The breeds and amounts of horses by breed in Sweden are presented in Figure 2 and in Norway in Figure 3.

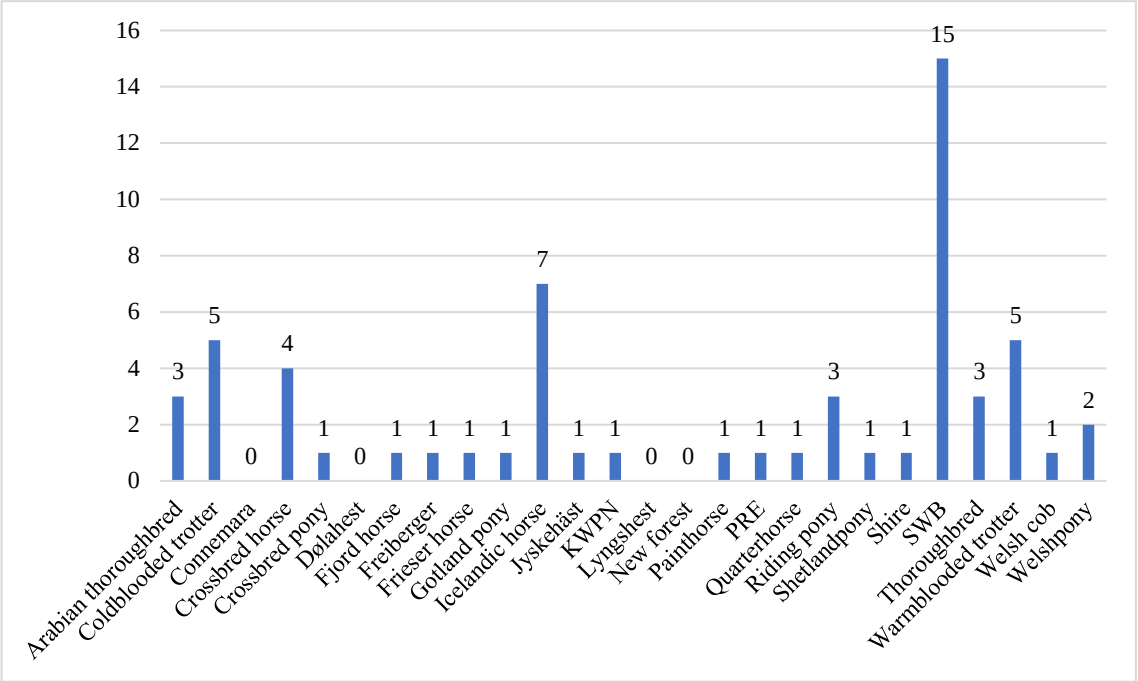

Figure 2. Breeds of the participating horses in Sweden.

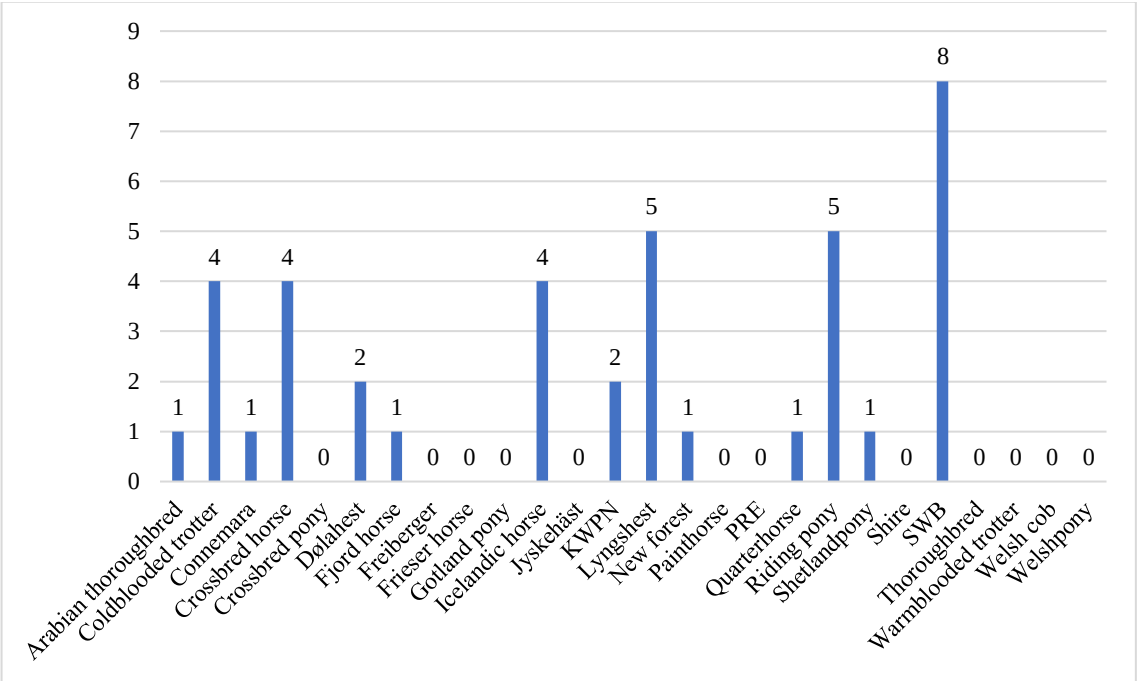

Figure 3. Breeds of the participating horses in Norway.

First sampling occasion was during October and November of 2016, second occasion during December and January of 2016-2017 and third during February and March of

2017. The participating horse owners were found by announcing the study at www.hästsverige.com which is an online portal owned by Swedish University of Agricultural Sciences (SLU). There was also a link to the announcement on Facebook to reach potential participants. In Norway the announcing was done via Norsk Hestesenter (2018), which describes itself to be the main organization for horse keeping and breeding as well as educating professionals in the equestrian field in Norway.

Every pair was matched with a case horse and a control horse and so a case and a control group of equal sizes were formed. The samples were collected by the horse owners during the three different sampling occasions of the study. The participants received detailed instructions for the procedure before the start of the study in order to have sampling performed as standardized as possible.

4.1.1 Participating criteria

There were participating criteria for the horses to increase the reliability and validity of the study. For the case and control horses to be comparable, they had to live in the same stable or loose housing system. They also had to be fed with the same roughage and be kept in the same pen or adjacent ones. The participants were to provide the researchers with complete information of the diet and possible medications that both control and case horses had been on for the last three months. None of the horses should recently have gone through major changes in their lives. For example, sudden changes in the diet or housing were exclusion criteria as they could affect the horse's gastrointestinal tract. Both horses should be healthy without any ongoing signs of infection, such as fever, flu, cough or gastrointestinal problems other than FFL in case horses. Both horses should also have recently been tested for parasites. This test could also be done during the study, but the owner would have to fund the test himself. The participating criteria was given to the horse owners in writing and a signed consent was required form participation.

4.1.2 Collection of samples

The faecal samples were collected by the horse owners. The instructions for collecting the samples were given along with the participating criteria in written form. It was instructed that both case and control horses should be sampled the same day and at the same time. The sample had to be from a fresh pile of faeces. When the owner had been accepted to being part of the study, they would receive packages for posting the samples as well as written instructions for collecting and packaging the samples. In connection with the sampling, measuring the rectal temperature of the horses was done to rule out ongoing infections as a cause for FFL. Also, once a month during the time the owners participated in the study, they were required to fill in a journal of faecal appearance and also note changes in for example stable environment, feeding and training.

4.2 Sample preparations

After arrival the faecal samples were stored in a freezer that was kept at temperature of -20 °C. Before doing the sample preparations the samples were taken out of the freezer to room temperature the day before. Usually 34-38 samples were taken out at the same time. The samples were not in any specific order and they could be of any sampling occasion of the study.

At the time of taking the samples out for thawing. Every sample was, if possible, divided into three smaller portions the next day. These would be used in different parts of the study. A digital scale with the accuracy of two decimals was used to weigh the samples. The first priority was to get a 30 g piece of every sample and to store them in plastic test jars that were 44 mm in diameter and 55 mm in height. These were then later used to analyse the dry matter content. The second priority was to get a big enough sample on a tray that would later be put in a drying cabinet, ground and used to measure the dry matter (DM) of the sample. These samples sizes varied, and the goal was to get samples that were at least 120 g in size but if this was not possible, smaller portions were used. Even much heavier samples were weighed in. This was because if the sample size sent to SLU was small, it was more convenient to use the whole amount at this point instead of sparing

the small amount that was not needed. The weight of an empty tray and the net weight of the sample were documented at this point. If the received sample was large enough, the remaining sample was saved in case it would be needed later. If by this point at least 140 g of the sample could be weighed, 20 g of that were put into zip lock-bags that were 100 x 150 x 0,05 mm in size. The samples in the zip locks would later be pressed with an industrial pressing machine to get liquid samples that could then be centrifuged and analysed for the total amount of liquid and possible particles, such as sand at the bottom of a test tube.

4.3 Measuring the dry matter content

To analyse the dry matter content in the samples, they were first dried in a drying cabinet in 55 °C for 18 hours. In practice this was done by placing the samples in the cabinet. The samples were taken out and weighed the following day, after drying and air equilibration.

4.4 Measuring the volume of free liquid and the amount of sand

4.4.1 Preparations

The 20 g samples that were kept in zip lock-bags and frozen afterwards were used to measure the liquid content in the faecal samples. The samples were taken out to room temperature the next morning and they got to thaw for about an hour before starting the pressing process.

4.4.2 Pressing the samples

After the samples were at room temperature, they could be pressed. This was done with an industrial pressing machine that could fit five samples at a time. The pressing was done by using compressed air and the process was all manual. Usually five samples were chosen at a time and the bags were pierced five times with a nail to create ten small holes

through which the pressed liquid could run. Funnels and 15 ml test tubes were placed under the containers to direct the liquid into the test tubes. After this the caps were put on the tubes, the machine was cleaned with paper towels, and the mobile parts were washed before the next batch would be pressed. The tubes were then placed in the fridge to wait for centrifuging.

4.4.3 Centrifuging the pressed liquid

The centrifuging was done in the afternoon following the pressing. The samples were centrifuged for one minute with the speed of 1000 rpm (rounds per minute). Radius was set at 0.5, brake at 9 and pre-cool at 10.

After the centrifuge had stopped the total amount of fluid was read as well as the volume of sand particles on the bottom of the test tube. This was done by eye reading of volumes. The results were documented in 0.01 ml accuracy.

4.5 Osmolality

4.5.1 Preparations

For osmolality measurements, liquid was pressed out of the samples with a hand-held manual fruit press. This liquid was then stored in a freezer until measure osmolality. The samples were thawed in lukewarm water for half an hour. 1,5 ml liquid was pipetted into an Eppendorf tube and then centrifuged in 20 000 x g for five minutes to get all solid particles to the bottom of the tube and all liquid to the top. 300 µl of the liquid was pipetted to a test cup that was used for measurement of osmolality using an osmometer.

4.5.2 Measuring the osmolality

The osmometer used was Advanced Osmometer, Model 3250, Advanced Instruments Inc., Norwood, MA, MOLEK AB. The buzzpoint was kept low, at 2000, as samples otherwise froze prematurely. The osmometer's calibration was tested in the beginning and end of each measurement day as well as with every 30th sample. This was done with calibrations liquids and distilled water. Calibration liquids used were Clinitrol 290, Reference solution, Advanced Instruments, INC. and Calibration standard, Advanced Instruments, INC.

4.6 Measuring the VFA content

4.6.1 Preparations

The faecal samples were prepared by centrifuging them for five minutes at 13000 g. Solid particles were sedimented at the bottom and 600 µl of the clear liquid on the top was used for analysis by high performance liquid chromatography (HPLC).

4.6.2 Chromatography

The HPLC system used consisted of Alliance 2795 Separations Module with Temperature control Module II range 0-150 ºC and 2414 RI Detector (Waters Assoc. USA). As pre- and separation columns the column package ReproGel H 9µ 300 x 8 mm (Dr. A. Maisch, Ammerbuch, Germany) was used. The following settings were used: mobile phase 5 mM H_2SO_4, flow rate 0.8 ml/min, column temperature 60 ºC and injection volume 20µl.

4.7 Measuring the pH

One part of the faecal samples was pressed with a potato press directly at their arrival to the laboratory. The liquid received from the pressing was then analysed with a pH 3110

ProfiLine pH meter (WTW, Germany) and two-point calibration with pH 4,01 and 7 was used.

4.8 Statistical analysis

The statistical analysis of the results was done by subjecting the data to ANOVA using the GLM procedure of SAS (SAS Institute Inc., Cary, NC, USA). Mean values, standard error values were calculated with the GLM procedure. The variables were for the affected and the unaffected horses. These two values were then compared to each other and the difference was examined. Also, the stable the horse lived in was taken into account when the GLM procedure was done. The stable was a block, minimizing random variation between the different stables. The differences in mean values were analysed within each sampling occasion which produced three different results for each mean value, standard error of mean as well as P-value.

The CORR procedure was used to get the Pearson correlation coefficients which were then used to study the correlations between the different chemical analysis variables. The correlations between the variables were examined within each sampling occasion resulting in three results for each variable.

5 RESULTS

5.1 Differences in faecal variables between case and control horses

5.1.1 First sampling occasion

Faecal samples from case horses had higher concentration of acetate (P = 0.032), iso-butyrate (P = 0.032) and total VFA (P = 0.043) compared to controls (Table 2). Case horses also tended to have higher volumes of liquid (P = 0.076) and sand (P = 0.066) and

higher concentration of n-butyrate (P = 0.070) in their faeces compared to controls (Table 2).

Table 2. Differences in variables between the case and control groups during the first sampling occasion. Differences are marked with bold text and tendencies toward difference (0.10 < P > 0.05) with italic text

Variable	**Unit**	**Control group**		**Case group**		
		x̄	SE	x̄	SE	P-value
DM[1]	%	18.7	0.05	18.7	0.05	0.523
pH		6.5	0.04	6.5	0.04	0.279
Liquid	ml	2.5	0.47	3.7	0.42	*0.076*
Sand	ml	0.1	0.01	0.1	0.01	*0.066*
Osmolality	mOsm/kg	152.1	5.48	158.9	5.60	0.392
Acetate	mmol/L	23.1	1.74	28.7	1.81	**0.032**
Propionate	mmol/L	8.1	0.48	8.8	0.50	0.335
Isobutyrate	mmol/L	0.8	0.05	0.9	0.05	**0.032**
n-Butyrate	mmol/L	2.2	0.19	2.8	0.20	*0.070*
Total VFA	mmol/L	39.0	2.68	47.1	2.79	**0.043**

[1]DM = dry matter

5.1.2 Second sampling occasion

Case horses tended to have a higher concentration of sand (P = 0.061) and n-butyrate (P = 0.077) in their faeces than the control group (Table 3).

Table 3. Differences in variables between the case and control groups during the second sampling occasion. Tendencies toward difference (0.10 < P > 0.05) are marked with italic text

Variable	**Unit**	**Control group**		**Case group**		
		x̄	SE	x̄	SE	P-value
DM[1]	%	18.6	0.13	18.3	0.13	0.247
pH	log	6.6	0.03	6.6	0.03	0.208
Liquid	ml	2.8	0.41	3.7	0.37	0.111
Sand	ml	0.0	0.01	0.1	0.01	*0.061*
Osmolality	mOsm/kg	150.5	3.82	145.7	3.90	0.379
Acetate	mmol/L	21.1	1.42	22.6	1.42	0.457
Propionate	mmol/L	7.0	0.36	7.7	0.36	0.221
Isobutyrate	mmol/L	0.8	0.04	0.8	0.04	0.618
n-Butyrate	mmol/L	1.5	0.15	1.8	0.15	*0.077*
Total VFA	mmol/L	34.2	2.08	37.2	2.08	0.321

[1]DM = dry matter

5.1.3 Third sampling occasion

In the third sampling occasion the only variable with tendency toward difference when the mean values of the two groups were compared was the pH value of the faeces sample. Horses with FFL had a higher pH than the unaffected horses (P = 0.088). These differences in the third sampling occasion are presented in Table 3.

Table 4. Differences in variables between the case and control groups during the third sampling occasion. Tendencies toward difference (0.10 < P > 0.05) are marked with italic text

Variable	**Unit**	**Control group**		**Case group**		
		x̄	SE	x̄	SE	P-value
DM[1]	%	18.4	0.08	18.2	0.08	0.211
pH	log	6.5	0.04	6.6	0.04	*0.088*
Liquid	ml	3.1	0.61	3.7	0.40	0.373
Sand	ml	0.1	0.01	0.1	0.01	0.173
Osmolality	mOsm/kg	134.1	6.33	142.8	6.20	0.328
Acetate	mmol/L	21.4	1.40	23.7	1.40	0.240
Propionate	mmol/L	6.8	0.39	6.8	0.39	0.938
Isobutyrate	mmol/L	0.8	0.04	0.9	0.04	0.117
n-Butyrate	mmol/L	2.0	0.19	2.2	0.19	0.341
Total VFA	mmol/L	35.2	2.29	38.5	2.29	0.320

[1]DM = dry matter

5.3 Correlations between the variables

Correlation coefficients between the variables when compared with one another during the first, second and third sampling occasions are presented in Tables 4, 5 and 6, respectively.

Correlation coefficients > 0.3 or < -0.3 between the different variables were found. The pH value was negatively correlated with all variables except for isobutyrate concentration.

Osmolality was negatively correlated with pH, and positively correlated to individual VFAs as wells to total VFA concentration. Individual VFAs were also found to correlate positively with one another and to total VFA.

The correlations that were found were consistent throughout all the three sampling occasions. The only thing differing between the correlations in the three sampling occasions was that a weak negative correlation coefficient was found between isobutyrate and pH in sampling occasions two and three, whereas in sampling one this could not be seen.

Table 5. Pearson correlation coefficients between faecal variables from the first sampling occasion

	pH	**Osmolality**	**Acetate**	**Propionate**	**Isobutyrate**	**n-Butyrate**	**Total VFA**
			Correlation coefficient				
pH		-0.44^{***}	-0.58^{***}	-0.53^{***}	-0.09	-0.51^{***}	-0.57^{***}
Osmolality	-0.44^{***}		0.84^{***}	0.79^{***}	0.54^{***}	0.65^{***}	0.84^{***}
Acetate	-0.58^{***}	0.84^{***}		0.91^{***}	0.58^{***}	0.79^{***}	0.99^{***}
Propionate	-0.53^{***}	0.79^{***}	0.91^{***}		0.56^{***}	0.75^{***}	0.94^{***}
Isobutyrate	-0.09	0.54^{***}	0.58^{***}	0.56^{***}		0.57^{***}	0.63^{***}
n-Butyrate	-0.51^{***}	0.65^{***}	0.79^{***}	0.75^{***}	0.57^{***}		0.86^{***}
Total VFA	-0.57^{***}	0.84^{***}	0.99^{***}	0.94^{***}	0.63^{***}	0.86^{***}	

*** $P < 0.001$

Table 6. Pearson correlation coefficients between faecal variables from the second sampling occasion

	pH	**Osmolality**	**Acetate**	**Propionate**	**Isobutyrate**	**n-Butyrate**	**Total VFA**
				Correlation coefficient			
pH		-0.40***	-0.64***	-0.61***	-0.29**	-0.49***	-0.62***
Osmolality	-0.40***		0.76***	0.74***	0.45***	0.70***	0.78***
Acetate	-0.64***	0.76***		0.88***	0.60***	0.81***	0.98***
Propionate	-0.61***	0.74***	0.88***		0.58***	0.84***	0.93***
Isobutyrate	-0.29**	0.45***	0.60***	0.58***		0.62***	0.66***
n-Butyrate	-0.49***	0.70***	0.81***	0.84***	0.62***		0.88***
Total VFA	-0.62***	0.78***	0.98***	0.93***	0.66***	0.88***	

** 0,01 < P > 0.001

*** P < 0.001

Table 7. Pearson correlation coefficients between faecal variables from the third sampling occasion

	pH	**Osmolality**	**Acetate**	**Propionate**	**Isobutyrate**	**n-Butyrate**	**Total VFA**
				Correlation coefficient			
pH		-0.44***	-0.47***	-0.44***	-0.26**	-0.43***	-0.47***
Osmolality	-0.44***		0.76***	0.80***	0.67***	0.59***	0.77***
Acetate	-0.47***	0.76***		0.92***	0.79***	0.83***	0.99***
Propionate	-0.44***	0.80***	0.92***		0.73***	0.75***	0.93***
Isobutyrate	-0.26**	0.67***	0.79***	0.73***		0.73***	0.82***
n-Butyrate	-0.43***	0.59***	0.83***	0.75***	0.73***		0.88***
Total VFA	-0.47***	0.77***	0.99***	0.93***	0.82***	0.88***	

** 0.01 < P > 0.001

*** P < 0.001

6 DISCUSSION

Free faecal liquid in horses has not been studied much, so there are not many studies where results are comparable with the results of this thesis. Both Ertelt and Gehlen (2015) and Kienzle *et al*. (2016) discussed the same problem and the reasons behind it. Comparisons between many other studies can only be a start of a larger discussion as the studies oftentimes were examining horses with problems such as diagnosed diarrhoea, while FFL is yet to be classified as an actual disease or health issue and it cannot yet be said if it is a form of chronic diarrhoea or not (Kienzle *et al*. 2016). The method of choice in this thesis was examining the faecal liquid of horses that were affected by FFL and unaffected horses. According to Zeyner *et al*. (2004) faecal liquid analysis is a valid method of examining the connection of at least diet and the microbial population in the equine large intestine.

6.1 Dry matter content

There was no difference in dry matter content in the faeces of the affected compared to unaffected horses in this study in any of the sampling occasions. This is logical as FFL does not automatically mean that the faeces are runnier but rather that liquid is separated from solid parts; the appearance of the faeces does not directly correlate with the dry matter content (Kienzle *et al*. 2016). The percentage of faecal water was measured using a method similar to the method used in this thesis to measure DM concentration in a study by Merritt and Smith (1980). The study examined foal heat diarrhoea. This included comparing the fresh weight of a faecal sample with a dried one. A higher water content was found to have a strong positive correlation with watery consistency of the faeces, where the range of consistency varied from watery to dry (Merrit and Smith 1980). In this thesis, higher water content in horses with FFL could only be seen in the first sampling. When compared to the study by Merritt and Smith (1980), this indicates that FFL truly is not a form of classical diarrhoea as it should increase the water content in the faeces. There are factors that are associated with the DM content of the faeces, higher hay consumption being a factor as it has seen to lead to relatively low DM content (Zeyner *et al*. 2004).

The mean value of faecal DM of test horses varied between 20.6 and 23.2 % in a study by Zeyner *et al*. (2004) examining the effects of hay intake and feeding sequence on variables in faeces and faecal water. This is higher than the results of this study where the mean value of DM concentration varied from 18.2 to 18.7 %. The differences in the results could be explained with the difference in experimental design and with the feeding of the horses and maybe even with the fact, that our study included horses that suffered from a health condition. Horses in the study by Zeyner *et al*. (2004) were used for a study on timing of feeding oats. Horses in the current study were fed in a variety of ways with haylage being the common factor, and this major difference in feeding could affect the results. In the study by Zeyner *et al*. (2004) the change in the diet was also a key factor when comparing the faecal parameters and this could too cause the difference in results. The study by Zeyner *et al*. (2004) was also smaller than this thesis's study, including only six horses. Larger sample size of the aforementioned study and therefore larger range of results, the lowest being as low as 11 %, is probably the reason for this difference.

6.2 pH value

The results of this study showed a tendency toward a higher pH value in the horses with FFL in the third sampling occasion. Different feeds have been shown to affect faecal pH in horses, as grains as the main concentrate in opposition to commercial pre-mixes has been associated with lower faecal pH (Williamson *et al.* 2007). This could be due to the fact that starch in grains may exceed the pre-caecal starch digestion capacity and result in more rapid fermentation in the hindgut which may cause more acidic faeces (Zeyner *et al*. 2004). In contrast, an increasing the amount of roughage in a horse's diet has been associated with higher faecal pH (National Research Council (U.S.) 1989). Increased quantity of roughage also leads to increased mastication and salivation which too buffers the acidity caused by VFAs and lactic acid in the stomach (Nicol *et al*. 2002).

The pH value of faecal water in horses was studied by Zeyner *et al*. (2004). The mean values varied between 6.36 and 6.68. This is in line with this thesis, the mean values having a range of 6.5-6.6, although the range in the aforementioned study is a bit wider.

It should be noted that faecal pH value lower than 6.2 has been associated with a challenging environment for cellulolytic and acetate-utilizing bacteria in the hindgut (Richard *et al*. 2006).

The mean pH value for geldings and colts was 6.41 and for mares and fillies 6.46 in the study by Williamson *et al*. (2007). In this study the subjects were intensively managed, healthy Thoroughbreds racehorses. The variation is quite alike the one got in this study, although the variation in the study group is smaller.

6.3 The volume of free liquid

Tendency toward difference was found in the volume of liquid in the faeces between the control and the affected group in the first sampling occasion. It should not be possible to explain this tendency with runnier faeces, as the problems with free faecal water are not characterized by classic diarrhoea but rather by two separate phases in the faeces. This happens when too much mechanical stress is applied to the structure and the liquid does not go back to the gel particles but stays outside (Kienzle *et al*. 2016). Also, this study's results implied that there is no difference in the dry matter content of the faeces of affected and unaffected horses. In other words, it could be that the liquid in the faeces of the horses affected by FFL is pressed out and stays separate of the solid particles more easily. The DM concentration does not take into account how water is attached to other substances in the sample, only how much water there is. The only thing that can be concluded when analysing the DM content is how much of the sample there is left after drying it. Because the volume of liquid in faecal samples in the current study was achieved by centrifuging the samples, the liquid volume is the free liquid in the sample. The relation between DM concentration and free liquid volume in equine faeces is unknown.

Also, there are other factors outside the gastrointestinal tract that contribute to the liquid content of faeces. Ambient temperature and humidity are examples of such factors, as they affect the horse's voluntary water intake which in its turn could have an effect on the faecal composition (Groenendyk *et al*. 1988). In this study only a tendency toward

difference – the horses with FFL having a higher mean value – between the case and control horses was found and only in one sampling occasion. This indicates that other factors, including external ones, may have a bigger effect on the volume of free liquid than the horse suffering from FFL.

6.4 The volume of sand

Horses can get sand in their intestine in several different ways. The sand could be eaten intentionally or accidentally while grazing or when fed off the ground (Ruohoniemi *et al*. 2001). This is normal, but when the amount of sand in the digestive tract gets too large, it might get impossible for the horse to clear the gastrointestinal tract of it. When enough sand is accumulated in the intestine, the horse may start to show clinical symptoms that may vary but are typically colic, diarrhoea and weight loss (Hart *et al*. 2013). It has been noted that it varies individually how much sand a horse can tolerate in their gastro-intestinal tract before starting to show symptoms (Ruohoniemi *et al*. 2001). The amount of faecal sand is sometimes used to examine intestinal sand, but correlations between the actual amount of sand in the gastrointestinal tract and amount of sand in faeces are poor. However, a total disappearance of sand in faeces could indicate one of two things: that there is no more sand in the gastrointestinal tract, or that the accumulation has gotten so distinct that the sand do no longer mix with the intestinal contents and is therefore not passed with faeces (Ruohoniemi *et al*. 2001). As much as 78 % of the sand intake has been noted to be excreted through the faeces during a 5 to 11 days (Hammock *et al*. 1998). This means that the remaining 22 % has the potential to accumulate in the intestine (Husted *et al*. 2005).

The amount of sand was one of the two variables showing consistency in difference in mean values in this thesis. During both first and second sampling occasion the sand volume showed a tendency of having a higher mean value for the FFL horses than for the control group. The larger amount of sand in faeces could be explained in many ways. One factor could be how the horses are fed; for example with a hay net or off the ground. Some feeds can also help to evacuate sand out of the intestine, such as psyllium and flaxseed

(Hotwagner and Iben 2008; Niinistö *et al*. 2014; Kara and Baytok 2017). This could make the management of the horse a crucial factor in developing or avoiding FFL.

6.5 Osmolality

Osmolarity is defined as the concentration of solute particles, in other words osmoles, per litre. Osmolality describes the same, but the unit used is number of osmoles in a kilogram. Measuring osmolarity or osmolality allows measuring of osmotic pressure of a solution. This is used to examine how different solvents are diffused across semipermeable membranes (Widmaier *et al*. 2008).

Mean osmolality of the faecal liquid of the horses in this thesis varied from 134.1 to 152.1 mOsm/kg in the control group and from 142.8 to 158.9 mOsm/kg in the case group. In comparison, a study by Masri *et al*. (1986) showed osmolarity ranging from 309 ± 35 to 434 ± 41 mOsm/litre in foals before the onset of foal heat diarrhoea. On day 0 of diarrhoea the osmolarity decreased significantly to 276 ± 22 mOsm/litre. After diarrhoea the osmolarity varied between 295 ± 42 and 336 ± 14 mOsm/litre. These results were therefore much higher than the results in this current study, where mean values of osmolality ranged from 134.05 to 158.87 mmol/kg. The results are directly comparable as the relation between the two units is 1:1. Explaining factors could be, for example, the age of the participating horses. A new-born foal's gastrointestinal system lacks microbes that full-grown horses have for a functioning hindgut fermentation (McKenzie III and Geor 2009). Foal heat diarrhoea typically occurs in the age of 6-14 days – in other words that is the probable age group of the study by Masri *et al*. (1986). The reason is suggested to be the developmental changes happening in the foal's digestive system during the first week of the foal's life (Masri *et al*. 1986). Suckling foals are able to digest fibre from two months of age indicating that they become herbivores at a young age. During the pre-weaning period, foals do not however have the carbohydrate-degrading capacity of an adult horse (Faubladier *et al*. 2013). Horses in this thesis also did not have diarrhoea, but even with the significant decrease in osmolarity during diarrhoea, the osmolality values were higher in the aforementioned study than in this thesis. It is also probable that different study methods could have affected the results; in both studies the samples were

frozen and centrifuged, but different machines, settings and amounts of faecal liquid could have had an impact on the results.

Osmolarity of equine faeces was measured also in the study by Merritt and Smith (1980). The differences between healthy horses and horses with chronic diarrhoea were examined. The mean osmolarity of the faeces of healthy horses was 219.4 mOsm/litre and of diarrhoeic horses 188.4 mOsm/litre. The mean values of osmolality of the unaffected horses in this thesis varied from 134.05 to 152.10 mOsm/kg and of the affected horses from 142.81 to 158.87 mOsm/kg. These values are low in comparison to the aforementioned study. The osmolarity of the faeces of the healthy horses was higher than for diarrhoeic horses in the study by Merritt and Smith (1980). No difference in osmolality between control and case horses was found in the current study. This is the expected outcome as problems with FFL are not characterized by diarrhoea.

6.6 VFA

The concentration and proportions of VFA in faeces can be used for descriptions of microbial activity and fluctuations in microbial activity in the proximal hindgut of the horse (Grimm *et al*. 2017).

This study's mean value of total VFA content varied from 34.2 to 39.0 mmol/litre in the control group and from 37.2 to 47.1 mmol/litre in the case group. This seems rather low when compared to a study by Masri *et al*. (1986); their total concentration before the foals got foal heat diarrhoea was 105 ± 12 mmol/litre and at its onset 55 ± 11 mmol/litre. After recovering from diarrhoea, the VFA concentration in the faeces of foals increased progressively to 111 ± 17 mmol/litre. The individual VFAs measured in the Masri *et al*. (1986) study were acetate, propionate, butyrate, isobutyrate, valerate and isovalerate. The fact that the study subjects were young foals is probably a contributing factor for the difference in results between the two studies. Also handling of the samples and study methods could have had an effect on the results.

In order to find out if a commercial product (EMP) was able to improve the gut health of horses, VFA were measured in equine faeces in a study by Gerstner and Liesegang (2018). EMP is a product containing montmorillonite–bentonite, whey and extracts from hop and absinthium (EmendoMOL PLUS (EMP), HealthBalance AG, Uzwil, Switzerland) and it was created to increase the intestinal health of horses. The study subjects were healthy horses, but behind it was an interest in the FFL problem. All the horses in the study were healthy and participated in both control and treatment group. No treatment effects were found in the faecal parameters. The mean value of acetate was 25.3 ± 3.2 mmol/L for the case group and 26.3 ± 5.9 mmol/L for the control group. The mean value of propionate was 4.1 ± 0.9 mmol/L for the case group and 4.1 ± 0.8 mmol/L for the control group. Finally, the mean value of isobutyrate was 0.9 ± 0.3 mmol/L for the case group and 0.9 ± 0.2 for the control group. Mean values of acetate, propionate and isobutyrate in this thesis are presented in tables 2, 3 and 4. It can be seen that these values are quite in line with the aforementioned study with the exception of propionate content that was higher in this thesis. A difference was found between the control and treatment groups when it came to acetate and isobutyrate but no difference was found when the effects of EMP were studied (Gerstner and Liesegang 2018). This indicates that EMP is probably not a suitable method of treating gastrointestinal problems, such as FFL, in horses.

Merritt and Smith (1980) studied the VFA concentration in equine faeces from healthy horses and horses with chronic diarrhoea. The healthy horses showed a mean value of total VFA of 52.7 µmol/ml while the mean value of diarrhoeic horses was 48.7 µmol/ml. It should be noted that in the study by Merritt and Smith (1980) the horses with chronic diarrhoea had lower total VFA content in their faeces than control horses, while this study showed a difference between the case and control groups only in the first sampling occasion and even then the horses with FFL had a higher concentration of VFAs in their faeces than the healthy horses. This further confirms the fact that being affected by FFL does not necessarily have the same effect on horses as having diarrhoea, but further studies are needed to increase knowledge about FFL in horses (Kienzle *et al*. 2016).

Some differences and tendencies toward differences were found between the case and control groups when it came to VFAs. The results were, however, inconsistent with difference and tendency toward difference appearing in two sampling occasions only in n-butyrate. This in mind, horses with FFL were found to have higher VFA contents than the unaffected horses. It could be the fact that horses with FFL absorb less VFAs from the feed or that they produce more VFAs than unaffected horses or it could be both. The difference could depend on the microbial population in the gastrointestinal track of the horse; the microbial population of a horse with FFL is probably different from an unaffected horse's one. This has not though been studied yet, so only speculations can be made.

6.7 Correlations

Correlations between the variables were found in this study. In all the sampling occasions the only variables that did not correlate with each other were pH and isobutyrate. Reasons for this are unclear, but factors such as butyrate not being as strong an acid as the other VFAs measured in this study, probably affect the results (Bruice 2016). This leads to it not to being able to have as big impact on the pH value. Also, the proportion of butyrate is small compared to other VFAs, which means that its effect is likely to be smaller than of the other VFAs.

In the study of Merritt and Smith (1980) a positive correlation was detected between VFA and osmolarity in the horses that were suffering from chronic diarrhoea. No correlation, on the other hand, could be seen between the VFA and osmolarity values in healthy horses. In our study the horses were not divided in groups by the presence when the correlations were examined but the division was done by sampling occasions instead. During all sampling occasions the individual VFAs as well as the total VFA content were found to correlate positively with osmolality. These results are in line with the results of the study by Merritt and Smith (1980) for horses with diarrhoea but not with the healthy ones. Despite this, the information about correlations between the variables that was got in this thesis could possibly be used to further study the reason behind FFL when paired

with discussion about the differences between the case and control horses, as the results are constant.

7 CONCLUSIONS

In most of the variables no differences could be seen between the horses with FFL and the unaffected horses. Most differences and tendencies toward difference were detected in the first sampling occasion, whereas only tendencies were found in the next two sampling occasions in a descendent manner.

Concentration of n-butyrate and volume of sand in faeces tended to be higher in case horses compared to controls in two of three sampling occasions. This could indicate that these two variables may play a role for horses developing FFL, or that the two variables are affected by the same unknown factor(s) that is causing FFL.

There was a tendency toward difference in the amount of faecal liquid in the first sampling occasion with the horses affected by FFL having a larger amount of liquid in their faeces. This difference could be explained with the ease of pressing and keeping the liquid out of the solid faeces. Alternatively, there could have been differences in the water contents of the faeces between the two groups but without further studies it cannot be said that this has anything to do with FFL as it could be explained with other factors such as management, environment, differences in the gastrointestinal system or other health issues the horse might have.

In the first sampling occasion, acetate, isobutyrate and total amount of VFAs were higher in the faeces of horses with FFL than in the faeces of the unaffected ones. The reason for this difference cannot be defined without further studies and might even be several factors contributing to this result. The reasons could lie in the microflora of the horses or in the

difference in production and absorption of VFAs. As differences between the groups when it came to these VFAs were found, it could indicate that VFAs might be a suitable way of studying and finding out possible reasons for FFL in horses. However, as the results were inconsistent, more studies are needed to verify this.

A tendency toward difference was noted in the pH value between the two groups in the third sampling occasion, the pH value of affected horses being higher than that of the unaffected ones. As even the highest results were quite in line with a couple of other studies and the difference was only noted in one sampling occasion, pH may not be the best way to start further examining FFL in horses.

Though not constant throughout all the three sampling occasions, there could also be seen other differences between the two groups of horses when the sampling occasions were examined individually. All mean values with differences were higher in the groups of horses with FFL than in the unaffected horses. It is though not known what the underlying reason for the difference is. It is probable that there is a difference in the microbial population of a horse with FFL and a one without the problem and this could affect the VFA content of the faeces. Management and intestinal peristalsis are probable reasons for the differences in the amount of bigger particles in faeces. This could also mean that the reasons for the differences between the results of the different sampling occasions could be explained with changes in management.

Yet there are other possible explanations for the different results between the sampling occasions. According to a study by Kienzle *et al*. (2016), season or change in weather might be factors when it comes to FFL. As the samples of the different sampling occasions were taken during different months, this could affect the results. The connection between FFL and season or changes in weather were not seen as significant in the study by Kienzle *et al*. (2016), but the phenomenon occurred in some of the horses according to the owners and thus it could be an affecting factor in a small scale. This could still be an explaining factor for why some of the horses with FFL seemed to show

smaller differences in the consistency of their faeces during the third sampling occasion when compared to the unaffected horses.

Almost all the variables, with only isobutyrate as an exception, were found correlating with one another during this study. As butyrate is important for the horses gastrointestinal health, this information could be used to detect the underlying reasons for FFL if differences between affected an unaffected horses are found.

FFL in horses has not been studied much yet and this particular thesis could only show that there are indications to differences between the faeces of horses with FFL when compared to horses without this particular problem. More studies are needed for reliable results.

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